

Steven M. Ruben
Appl. No. 10/662,429

Date: 8/30/95
To: Gordon Moore
From: Alem Truneh
K.B. Tan
Christopher Eichman
Manjula Reddy
Subject: Monthly Report / October 1995

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Ruben EXHIBIT #77

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Ruben EXHIBIT 2077
Ruben v. Wiley et al.
Interference No. 105,077
RX 2077

TNF/TNFR

- Second SB/HGS Joint Meeting (first since formation of TNF/TNFR Team) held in October. Team has agreed to conduct regularly joint meetings every 3 months.
- Team has so far identified at least 12 new genes from the ATG/HGS database which belong to these classes including:
 - 4 ligands
 - 8 receptors (includes 3 splice variants)
- Other putative novel genes and splice variants at various stages of evaluations for authenticity and/or novelty
- Most of the class 1 genes in this family are also available as full length genes at HGS
- Preliminary studies of RNA expression of one TNF-like and 3 TNF receptor-like genes have been completed. TNF α and Ox40 were included as controls (class 1 gene of TNF receptor superfamily). The novel genes under study have been renamed (Table 1) as TL (TNF Ligand superfamily) or TR (TNF Receptor superfamily). A summary of the studies is presented in Table 2, which lists the cells or tissues that show high levels of gene expression.
- Total RNA from the following sources was prepared and examined:
 - 11 human tissues (including all major organs and hematopoietic tissues)
 - 23 human cell lines
- Total RNA from rodents including mouse and rat tissues has also been prepared
- TR2 has been expressed as Ig-fusion protein
 - 2 mgs have been purified: currently being using for biochemical analysis, preliminary binding studies and for generation of mAbs

- Preliminary binding studies conducted by flow cytometry using over a dozen human cell lines. However, definitive conclusion on binding characteristics can not be made until sufficient Ig-fusion material has been generated to prepare cleave product for blocking controls (i.e. to exclude possibility of binding via Fc portion of molecule).

Table 1. TNF Ligand and Receptor Superfamily Genes

<i>Name</i>	TL1	TL2	TR1	TR2	TR3	OX40
<i>HGS code</i>	250630	413412	195197	103902	231556	117992
<i>Project ID</i>	HUVE091	HTPAN08	HSABH13X	HTXBS40	HTTBN61	HT4SD09
<i>SB code</i>	ATG 339	ATG 343	ATG 348	ATG 363	ATG 338	ATG 342
<i>Library</i>	umbilical vein	pancreatic tumor	fetal lung fibroblast line	T cells	testis tumor	activated T cells
<i>Class</i>	class 5	class 3	class 2	class 2	class 5	class 1
<i>Match</i>	unknown				unknown	

Table 2. SUMMARY OF RNA EXPRESSION STUDIES

Gene library	Myeloid cells	B cell	T cell	Osteo-sarcoma	Tissues	Primary cells	Multiple forms
TNF α	HL60	REH (pre B)			Heart	CD4+	
TL2 pancreatic tumor	KG1a		Jurkat		Heart Bone marrow	CD4+ CD19+	Yes
TR1 fetal lung fibroblast				MG63	Kidney		
TR2 T cell	KG1a		Jurkat		Lung, Thymus Spleen Bone marrow	CD4+ CD8+ CD19+	
TR3 testis tumor	KG1a				Thymus	CD4+	Yes
OX40 activated T cell	KG1a		Jurkat			CD4+	

TNF and TNFR research effort

Preliminary studies of RNA expression of one TNF-like and 3 TNF receptor-like genes have been completed. As controls, we have included TNF α and Ox40 (class 1 gene of TNF receptor superfamily) in our studies. The novel genes under study have been renamed (Table 1) as TL (TNF Ligand superfamily) or TR (TNF Receptor superfamily). The data on studies with human and rat tissues and fresh human cells are presented in Table 2. Studies with tissue culture cell lines are presented in Table 3. A summary of the studies is presented in Table 4, which lists the cells or tissues that show high levels of gene expression.

Table 1. TNF Ligand and Receptor Superfamily Genes

<i>Name</i>	TL1	TL2	TR1	TR2	TR3	OX40
<i>HGS code</i>	250630	413412	195197	103902	231556	117992
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<i>Library</i>	umbilical vein	pancreatic tumor	fetal lung fibroblast line	T cells	testis tumor	activated T cells
<i>Class</i>	class 5	class 3	class 2	class 2	class 5	class 1
<i>Match</i>	unknown gene or protein	known non-human gene or protein	known human protein	known human protein	unknown gene or protein	known human gene

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Table 2. TNF Ligand and Receptor Superfamily Gene Expression

TISSUES and CELLS	TNF α (class 1)	TL2 413412 HTPAN08 ATG 343 pancreatic tumor (class 3)	TR1 195197 HSABH13X ATG 348 fetal lung fibroblast line (class 2)	TR2 103902 HTXBS40 ATG 363 T cell (class 2)	TR3 231556 HTTBN61 ATG 338 testis tumor (class 5)	OX40 117992 HT4SD09 ATG 342 activated T cells (class 1)
HUMAN						
Brain	-	-	-	-	-	-
Heart	++	++	+	+	-	-
Lung	\pm	+	+	++	\pm	-
Thymus	-	-	-	++	++	-
Spleen	\pm	\pm	-	++	+	-
Liver	-	-	-	-	-	-
Kidney	-	+	++	+	-	-
Small Intestine	\pm	-	-	+	\pm	-
Prostate	-	\pm	-	+	-	-
Skeletal Muscle	\pm	-	-	-	-	-
Bone Marrow	\pm	++	-	++	\pm	-
PBL, CD19+	\pm	++	-	++	\pm	-
PBL, CD8+	\pm	+	-	++	-	-
PBL, CD4+ (activated)	+++	+++	\pm	++	++	++
RAT						
Brain	-	-	-	-	-	-
Heart	++	-	-	-	-	-
Lung	-	-	-	-	-	-
Thymus	-	-	-	-	-	-
Spleen	-	-	-	-	-	-
Kidney	+	-	-	-	-	-
Small Intestine	-	-	-	-	-	-

- = not detected, \pm to +++ = increasing amounts of RNA detected.

Table 3. TNF Ligand and Receptor Superfamily Gene Expression

CELL LINES	TNF α (class 1)	TL2 413412 Pancreatic tumor (class 3)	TR1 195197 Lung fibroblast (class 2)	TR2 103902 T cell (class 2)	TR3 231556 Testis tumor (class 5)	OX40 117992 Activated T cells (class 1)
<i>Bone Marrow Stromal</i>						
TF274	-	\pm	+	-	-	-
<i>Osteosarcoma</i>						
MG63	-	-	++	-	-	-
HOS (TE85)		\pm	\pm	-	-	-
<i>Hematopoietic: Erythroid</i>						
K562			-			
<i>Hematopoietic: Myeloid</i>						
KG1a	-	++	-	+	++	+
KG1	-	++	-	\pm	+	\pm
PLB985			-		-	
HL60	++	\pm	-	-	-	-
U937			-	\pm	-	
THP-1	\pm	\pm	\pm	+	-	-
<i>Hematopoietic: B-Lymphocyte</i>						
REH (Pre B)	+	-	-	\pm	-	-
BJAB (IgM)			-	-	-	
Raji (IgM)	\pm	-	-	\pm	-	-
IM-9 (IgG)			-	-	-	
<i>Hematopoietic: T-Lymphocyte</i>						
Sup-T1			-	-	-	
Jurkat	-	+	-	+	-	+
H9				+		
Molt-3	-	-	-	\pm	-	-
<i>Endometrial Carcinoma</i>						
RL95-2			-	-	-	
<i>Breast Cancer</i>						
MCF7	-	-	\pm	-	-	-
<i>Colon Cancer</i>						
BE			-	-	-	
HT29			-	-	-	
<i>Neuroblastoma</i>						
IMR32				-	-	

no entry = not tested, - = not detected, \pm to ++ = increasing amounts of RNA detected.

Table 4. SUMMARY OF RNA EXPRESSION STUDIES

Gene library	Myeloid cells	B cell	T cell	Osteo-sarcoma	Tissues	Primary cells	Multiple forms
TNF α	HL60	REH (pre B)			Heart	CD4+	
TL2 pancreatic tumor	KG1a		Jurkat		Heart Bone marrow	CD4+ CD19+	Yes
TR1 fetal lung fibroblast				MG63	Kidney		
TR2 T cell	KG1a		Jurkat		Lung, Thymus Spleen Bone marrow	CD4+ CD8+ CD19+	
TR3 testis tumor	KG1a				Thymus	CD4+	Yes
OX40 activated T cell	KG1a		Jurkat			CD4+	

EXPRESSION OF TNF-LIKE PROTEIN

HGS clone 413412 = HTPAN08

Objectives:

- Express as fusion protein in E. coli--to be used for raising antibodies
- Express in soluble form in E. coli or other systems--to be used for receptor binding and activity assays

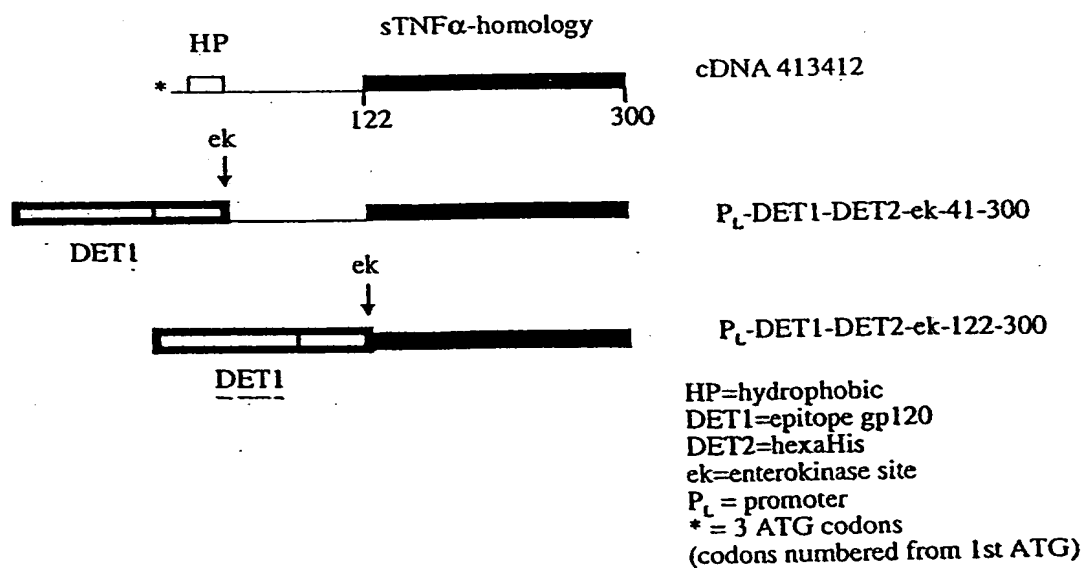
Accomplishments:

- Plasmid DNA from HGS strain (purified by K. B. Tan) was sequenced.

The DNA sequence in the open reading frame agreed with that reported by HGS.

There was a 1 bp difference upstream of the first of 3 closely spaced in-frame ATG codons, which makes it more likely that the first ATG is the translation initiator codon (TTCATGG in HGS sequence is ATCATGG in SB sequence; the latter is in good agreement with Kozak consensus upstream sequence)

- Two fusion constructs are being made for E. coli expression; both have the potential for release of soluble protein following enterokinase digestion.



TNFRL2

The putative transmembrane domain of translated TNFRL2 sequence was determined by hydrophobicity using the method of Goldman et al for identifying nonpolar transbilayer helices. The region upstream of this transmembrane domain, encoding the putative leader peptide and extracellular domain, was chosen for the production of an Fc fusion protein. Primers were designed to PCR this fragment from HGS 325532 with the addition of a BglII site and a Factor Xa protease site at the 3' end. This fragment was to be cloned into the COSFclink plasmid to produce the Fc fusion.

However, HGS 103902 was received instead of HGS 325532. Upon sequencing, the former was found to have none of the 5' untranslated sequence that was used to design the upstream PCR primer. In addition, the start Met codon of TNFRL2 was replaced by a His codon. A new upstream primer was designed based on the sequence of 103902 which replaced the His codon with a start Met codon and was compatible with the previously designed downstream primer. PCR with this primer pair resulted in one band of the expected size. This was cloned into COSFclink to give the TNFRL2Fclink plasmid.

COS cells were transiently transfected with TNFRL2Fclink and the resulting supernatants were immunoprecipitated with protein A agarose. Western blot analysis of the immunoprecipitate using goat anti-human Fc antibodies revealed a strong band consistent with the expected size for glycosylated TNFRL2Fc (greater than 47.5 kD). A transient COS transfection was requested from the Small Scale Cell Culture Lab (BPD) and the resulting supernatant is being purified by Protein Biochemistry. This protein will be sent to Steve Holmes to immunize mice following DNA injection for the production of mAbs (see below).

CHO E1a (ACC 317) cells were transfected with TNFRL2Fclink to produce stable cell lines. Five lines have been chosen for expansion by dot blot analysis and are currently being adapted to shake flasks. Three aliquots of each line have been frozen down.

Sequence analysis of TNFRL2Fclink and HGS 103902 indicated that both contained two nucleotides that conflicted with other TNFRL2 sequences including that found in the full length report. One of these changes (T instead of C) resulted in a Phe codon as opposed to a Ser codon, while the other nt change (C instead of T) was silent. Mark Hurle determined that HGS sequence data for the EST's of TNFRL2 show T and C are represented at both positions. Another full length TNFRL2 clone, HGS 338804, will be sequenced in-house for comparison. It was also noted that the HGS sequences for 338804 and 338805

differ from the others by the replacement of a G with an A, resulting in the replacement of an Arg codon with a Lys codon.

Originally the TNFRL2FcLink plasmid was to be sent to Steve Holmes for the initial injection of mice for the production of mAbs. However, his data indicated that a high percentage of anti-Fc mAbs were being generated following DNA immunization with ALK5-Fc plasmid. The Fc fragment was therefore removed from TNFRL2FcLink and the resulting frame shift introduced a stop codon. Thus, the C terminus of the extracellular domain of TNFRL2 is followed by only 2 aa (RS) in this construct (TNFRL2exLink). A clone with the correct sequence through the ligated ends is being maxiprep'd for shipment to Steve Holmes.

Immediate future:

- for antibody production:
 - TNFRL2exLink to Steve Holmes for first immunization for mAbs
 - small-scale COS prep:
 - second and third immunizations for mAbs
 - immunizations for polyclonal antibodies
- stable transfectants of CHO E1a cells (ACC 317):
 - adapt to shake flasks
 - perform Western analysis on supernatants
 - choose line with highest expression
 - begin FACS experiments

sTNFRL1

The sequence of sTNFRL1 derived from the HGS full length report shows no transmembrane region by hydrophobicity plot (Goldman et al., see TNFRL2 above). Neither K. B. Tan nor I were able to purify plasmid from the clone of HGS 195197 received from BPS. K. B. subsequently received 195197 DNA directly from HGS and transformed it into XL1Blue cells. This clone is currently being sequenced in-house prior to the design of PCR primers for the production of an Fc fusion.

Immediate future:

- analyze sequence data
- construct sTNFRL1FcLink and sTNFRL1exLink
- proceed as for TNFRL2

TNFRL2 / Immediate future:

- mAb production
 - TNFRL2exlink to Steve Holmes for first immunization for mAbs
- small-scale COS prep:
 - second and third immunizations for mAbs
 - immunizations for polyclonal antibodies
- stable transfectants of CHO E1a cells (ACC 317):
 - adapt to shake flasks
 - perform Western analysis on supernatants
 - choose line with highest expression
 - begin FACS analysis

sTNFRL1

- Sequence of sTNFRL1 derived from the HGS full length report shows no transmembrane region by hydrophobicity plot
- Neither K. B. Tan nor Sally were able to purify plasmid from the clone of HGS 195197 received from BPS
- 195197 DNA subsequently received by K. B. directly from HGS transformed into XL1Blue cells
- Clone currently being sequenced in-house prior to the design of PCR primers for the production of Ig-fusion

Immediate future:

- analyze sequence data
- construct sTNFRL1Fc link and sTNFRL1ex link
- proceed as for TNFRL2

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